The Role of Particulate Matter-Associated Zinc in Cardiac Injury in Rats

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Acknowledgments

The authors express appreciation to Mr. James Lehmann and Mr. Darrell Winsett of the US EPA for intratracheal instillations. We thank Drs. James Samet, Adriana LaGier, Gary Hatch and Linda Birnbaum (US EPA) for critical review of this manuscript. This work was supported in part by U.S. EPA/University of North Carolina Training Agreement, CT829471 and CR829522. The authors declare they have no competing financial interests.

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The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and the policies of the agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Short Title

Pulmonary Exposure to Zinc and Cardiac Effects

Keywords

Aconitase, Air pollution, Cardiac gene expression profile; Mitochondria, Particulate matter, Zinc

Abbreviations

BALF, bronchoalveolar lavage fluid

cRNA- complimentary ribonucleic acid

DEPC, diethylpyrocarbonate

DNA, deoxyribonucleic acid

dsDNA, double stranded deoxyribonucleic acid

mRNA, messenger ribonucleic acid

MSH, Mount St. Helen's ash

MT-1, metallothionein-1

PM, particulate matter

PM-HD, particulate matter at high dose level

PM-LD, particulate matter at low dose level

PM-L, particulate matter, saline leachable fraction

Q-PCR, quantitative polymerase chain reaction.

RNA, Ribonucleic acid

WKY, Wistar Kyoto

ZT-2, zinc transporter-2

Outline of the manuscript

Abstract

Background: Exposure to particulate matter (PM) has been associated with increased cardiovascular morbidity; however, causative components are unknown. Zinc is a major element detected at high levels in urban air.

Objective: We investigated the role of PM-associated zinc in cardiac injury.

Methods: Male 12-14 wk old Wistar-Kyoto rats were repeatedly exposed intratracheally (once/wk x 8 or16 wk) to either saline (control); PM having no soluble zinc (Mount St. Helen's ash; MSH); whole combustion PM suspension containing 14.5 μg/mg of water-soluble zinc at high (PM-HD) and low dose (PM-LD), the aqueous fraction of this suspension (14.5 μg/mg of soluble zinc) (PM-L), or zinc sulfate (8 wk rats received double the concentration of all PM components of 16 wk).

Results: Pulmonary inflammation was apparent in all exposure groups when compared to saline (8 wk>16 wk). PM, with- or without-zinc, or zinc alone caused small increases in focal subepicardial inflammation, degeneration and fibrosis. Lesions were not detected in controls at 8 but were noted at 16 wk. We analyzed mitochondrial DNA damage using QPCR and found that all groups except MSH caused varying degree of damage relative to control. Total cardiac aconitase activity was inhibited in rats receiving soluble zinc. Expression array analysis of heart tissue revealed modest changes in mRNA for genes involved in signaling, ion channels function, oxidative stress, mitochondrial fatty acid metabolism, and cell cycle regulation in zinc but not MSH-exposed rats.

Conclusion: These results suggest that water-soluble PM-associated zinc may be one of the causal components involved in PM cardiac effects.

Introduction

Although human exposure to airborne particulate matter (PM) is associated with adverse cardiovascular effects (Pope et al. 2004), the specific causative components or sources have not been identified. Ambient PM is physicochemically heterogeneous containing significant quantities of metals, including iron, aluminum, silica, zinc and copper; the levels of these elements vary depending on the geographical location and the local sources (Harrison and Yin 2000; Kodavanti et al. 2005; Ostro et al. 2007; Schwar et al. 1988). Zinc is a ubiquitous PM metal reaching nearly 27 $\mu g/m^3$ airborne concentration in industrial areas of developing countries (Harrison and Yin 2000). Tire- and brake-wear may also contribute to the near-road atmospheric concentration of zinc (Adachi and Tainosho 2004; Councell et al. 2004).

Zinc is an essential nutrient required for the maintenance of cell growth, immune maturation and reproduction, and is known to function as an antioxidant via induction of metallothioneins (Maret 2000; 2004; Maret and Sandstead 2006). Although milligrams of quantities of zinc are ingested daily in foods and with vitamin supplements, at slightly higher than physiological level, it causes cardiovascular and neuronal toxicity (Dineley et al. 2003; Maret 2000; 2006). While there are few reports suggesting zinc-mediated cardiac toxicity (Evangelou and Kalfakakou 1993; Klevay et al. 1994), its multifaceted effects on mitochondrial respiration (Ye et al. 2001), calcium homeostasis (Hershfinkel et al. 2001; Maret 2001), sulfur-metal coordination (Maret 2004), intracellular signaling (Samet et al. 2003), myocyte ion channels (Graff et al. 2004), and competition with other essential metals (Klevay 2000; Labbe and Fischer 1984) are well documented and may portend a risk of toxicity.

PM-associated water-soluble metals, including zinc, can be absorbed via the pulmonary vasculature upon deposition, potentially reaching cardiac tissue at high concentrations (Gilmour et al. 2006a; Wallenborn et al. 2007) before it is sequestered in the liver. We recently reported that long-term, episodic inhalation exposure to zinc enriched combustion particles, similar to some ambient PM (Adamson et al. 2000), caused myocardial injury in the Wistar Kyoto rat (Kodavanti et al. 2003). We further demonstrated that a bolus pulmonary exposure to zinc causes marked changes in cardiac gene expression that is reflective of the impairment in mitochondrial respiration, cell signaling, Ca^{2+} homeostasis and ion channel function (Gilmour et al. 2006b). In this study, we have hypothesized that particle-associated, water-soluble zinc is one of the causative PM components responsible for myocardial effects including oxidative stress and altered cell signaling resulting from protracted exposures. We compared the toxicities of PM with or without zinc, the water-soluble fraction of zinc-containing PM, and soluble zinc alone. Particle exposures, with or without zinc, increased the incidence of cardiac lesions to a small extent, perhaps as a result of long-term pulmonary inflammation. However, only the soluble zinc, and to some extent zinc containing PM suspension, or the leachate fraction caused moderate inhibition of cardiac total aconitase activity, mitochondrial DNA damage, and changes in cardiac gene expression. These changes in gene expression are consistent with alterations in cell growth, signaling, mitochondrial fatty acid metabolism, ion channel function and overall oxidative stress. Thus, PM-associated zinc may be one of the causative components of ambient PM responsible for cardiac effects.

Methods and Materials

Animals

Healthy, male, 12-14 week old Wistar Kyoto (WKY) rats were purchased from Charles River Laboratories, Raleigh, NC. All rats were maintained in an isolated animal room in an AAALAC approved animal facility (21 ± 1°C, 50 ± 5% relative humidity, 12 h light/dark cycle) for one to two week quarantine and non-exposure periods. The rats were housed in plastic cages with beta chip bedding. All animals received standard (5001) Purina rat chow (Brentwood, MO) and water ad libitum. The EPA's Animal Care and Use Committee approved the protocol for the use of rats in these studies. Animals were treated humanely and with regard for alleviation of suffering.

Rationale for selection of PM samples, exposure methods and concentrations

We have recently reported that a 16 weeks episodic inhalation exposure of rats to oil combustion PM containing water soluble zinc caused myocardial injury (Kodavanti et al. 2003). We wanted to determine if cardiac injury was caused by zinc leached from PM or was secondary to pulmonary inflammation that may occur as a result of deposition of PM in the lung. Therefore, we designed a study protocol, which used 6 groups (n=8) of male WKY rats receiving different components of PM (Table 1) for each of two time points (8 and 16 weeks). Group 1 received Saline to serve as a control. Group 2 received Mount St. Helen's ash (MSH), which does not contain any water-soluble zinc or other metals (McGee et al. 2003) such that we can delineate any cardiac effect secondary to pulmonary inflammation/injury following deposition of these particles as these fine mode particles themselves are not likely to translocate to the heart. Group 3 received whole saline suspension at high dose (PM-HD) of the same fugitive oil combustion particle sample used in the previous study and contained insoluble

components plus water-soluble zinc (Kodavanti et al. 2002; 14.5 μg/mg of zinc) and also a small amount of water-soluble nickel (3.0 μg/mg). The elemental composition of this PM is comparable to the previously used Ottawa urban PM (Adamson et al., 2000). Group 4 also received this particle sample but at half the dose of group 3 (PM-LD). Group 5 received leachable fraction (saline-soluble) of PM-HD devoid of any solid material but containing the soluble components of zinc and nickel (PM-L). Finally, Group 6 received saline solubilized zinc sulfate (zinc) at a concentration of zinc equivalent to Groups 3 or 5. This design allowed us to test if cardiac injury was due to soluble zinc or secondary to PM-induced pulmonary inflammation.

Since it is technically challenging to use inhalation methodology for exposure of rats to all these fractions concurrently, repeated weekly intratracheal instillations were used. We have shown that the instillation of a similar fly ash can give very similar pulmonary outcome after a single instillation if the instillation and inhalation doses are matched (Costa et al. 2006). Particle dose was based on our previous inhalation study, which used the same PM and led to cardiac injury (Kodavanti et al. 2003). Our previous study included 10 mg/m^3 of inhaled PM, 6h/d, 1d/week for sixteen weeks. The total lung deposition fraction was assumed to be 0.32 and the cumulative lung dose of PM over the 16 weeks period was calculated to be 5.53 mg for a 300 g rat (18.43 mg/kg body weight). This total deposition amount was divided by 16 for each weekly intratracheal dose in the low dose particle (PM-LD) group 4 (1.15 mg/kg). To assure sufficient particle exposure to cause cardiac injury from fractions of the whole particle suspension, group 3 (PM-HD) received double this dose (2.30 mg/kg). The same mass dose of MSH was used for group 2 animals (2.30 mg/kg). A saline-leachable fraction devoid of solid components was prepared from the particle suspension (2.30 mg/kg) and given to

group 5 (PM-L). Zinc concentration in this water-soluble fraction of particle was 14.5 μg/mg and therefore, group 6 animals received zinc sulfate solution amounting to 33.4 μg zinc/kg body weight (the amount of water-soluble zinc present in 2.3 mg PM). Rats in the 8 weeks study received 8 weekly instillations but with all the components at double the concentration than those used for 16 weeks. Eight weeks exposure was used to determine if cardiac effects can be apparent within a short period of time, such that future studies can be planned using this timeframe when material is limited. The rationale for doubling the concentration for 8 weeks was to assure detection of cardiac injury based on the theoretical dose for 16 weeks that was needed for detectable injury in our earlier study (Kodavanti et al. 2003). Because the concentration of the weekly PM doses were same for 8 weeks PM-LD group and 16 weeks PM-HD groups, we were able to determine the progression of effects with repeated PM exposures.

Intratracheal instillation

We used WKY rats for this study because this strain has shown lower background cardiac lesions than Sprague Dowley rats (Kodavanti et al. 2003), although sporadic myopathy is apparent (Kuribayashi, 1987). In our previous study, this strain appeared to be specifically sensitive to PM cardiac injury due to low background cardiac spontaneous lesions (Kodavanti et al. 2003). Rats were randomized by body weight into 6 groups (n=8) for each time point. Each particle sample was suspended in sterile saline at the desired concentration and was mixed continuously for 20 min prior to their use. To prepare the soluble fraction, the PM-HD was centrifuged at 12,000 x g for 10 min and the supernatant fluid filtered through a Teflon syringe filter unit (0.25 μm pore size). This filtered fraction was termed as the leachable fraction (PM-L). Similarly, zinc sulfate was dissolved in sterile saline to give 33.4 μg zinc per ml. All fractions, including saline

were intratracheally instilled once per week at 1 ml/kg under halothane anesthesia (Costa et al. 1986).

Necropsy, sample collection, and analysis

We selected a 48 h time point following the last instillation for necropsy to minimize impact of the last intratracheal instillation and maximize detection of subchronic outcomes. Two days following the last instillation, rats were anesthetized with an overdose of sodium pentobarbital (50-100 mg/kg, ip). Blood was drawn and the heart was removed, blotted dry, weighed and cut into two mid-longitudinal halves, one for enzyme assay and RNA/DNA isolation and second for histology. The right ventricle was discarded from the first half and portions of the left ventricle plus septum were snap-frozen in liquid nitrogen and retained for enzymes activity analysis and RNA isolation.

The second half of the heart was fixed in 10% neutral buffered formalin. The 1st, 3rd, 5th and 8th consecutive sections were stained with hematoxylin and eosin (H&E) and examined microscopically without prior knowledge of the treatment groups. A more detailed description of histology assessment is provided in the Supplemental Material.

Immediately following removal of the heart, the trachea was cannulated, left lung was tied and the right lung lavaged using Ca^{++}/Mg^{++} free phosphate buffered saline (pH 7.4) as previously described (Kodavanti et al., 2002). Aliquots of bronchoalveolar lavage fluid (BALF) were used to determine total cell counts with a Z1 Coulter Counter (Coulter, Inc., Miami, FL).

RNA isolation

Total heart RNA was isolated from tissues snap-frozen in liquid nitrogen using TriReagent (Sigma, St Louis, MO). RNA was further purified with Qiagen Rneasy mini

columns (Qiagen, Valencia, CA) and resuspended in 50 μl diethylpyrocarbonate (DEPC)-treated water according to the manufacturer's protocol. RNA quantity was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All samples had a 28S/18S ratio ≥ 2.0 and were stored at –80^{o}C before shipment on dry ice to Expression Analysis Inc. (Durham, NC) or their use in real-time PCR.

Microarray target preparation and hybridization

Expression Analysis Inc. (www.expressionanalysis.com; Durham, NC) performed RNA target preparation and hybridization to the Affymetrix GeneChip Rat 230A microarray containing 15923 probe sets and expressed sequence tags (Affymetrix Inc., Santa Clara, CA) according to the "Affymetrix Technical Manual". A brief description of the process for cRNA synthesis, hybridization, visualization and quantification are described elsewhere (Gilmour et al. 2006b). Fluorescent images were detected in a GeneChip Scanner 3000 (Affymetrix) and expression data was extracted using the default setting in the microarray Suite 5.0 software (Affymetrix). For microarray purposes, four biological replicates were collected for each group.

Real-time polymerase chain reaction (PCR)

In order to confirm the Affymetrix gene array data, real-time quantitative PCR was performed for metallothionein-1 (MT-1), and zinc transporter-2 (ZT-2) using heart RNA derived from 8 weeks exposure group, essentially as described previously (Gilmour et al. 2006b). Zinc exposure caused a 30% increase in MT-1 mRNA expression over saline control which is consistent with the data obtained in the microarray in the present study (insignificant in each case). No significant increases were noted in ZT-2 mRNA expression by either method.

DNA isolation and mitochondrial DNA damage analysis using quantitative polymerase chain reaction (Q-PCR)

DNA was extracted from left ventricular tissue of 8 weeks exposure group using a genomic DNA extraction kit (Qiagen, Chatsworth, VA) according to the protocol supplied with the kit. DNA was quantified using the PicoGreen® dsDNA Quantitation Kit (Invitrogen Corp., Carlsbad, CA). Q-PCR was done using a protocol described previously (Ayala-Torres et al. 2000; Santos et al. 2002), except for the quantification of PCR products which was done using PicoGreen dye. The primer sequences used were as follows: for the 12.4-kb nuclear gene, clusterin 5′-AGA CGG GTG AGA CAG CTG CAC CTT TTC-3′ and 5′-CGA GAG CAT CAA GTG CAG GCA TTA GAG-3′; for the 13.4-kb mitochondrial genome, 5′-AAA ATC CCC GCA AAC AAT GAC CAC CC-3′ and 5′-GGC AAT TAA GAG TGG GAT GGA GCC AA-3′; and for the 235-bp mitochondrial fragment, 5′-CCT CCC ATT CAT TAT CGC CGC CCT TGC-3′ and 5′-GTC TGG GTC TCC TAG TAG GTC TGG GAA-3′. DNA lesion frequencies were calculated as described (Mandavilli et al. 2000). Briefly, the amplification of damaged samples (A_D) was normalized to the amplification of a non damaged control (A_O), resulting in a relative amplification ratio. Assuming a random distribution of lesions and using the Poisson probability mass function equation [f [x!] = $e^{-\lambda}\lambda^x/x$], where λ = the average lesion frequency for the non-damaged template (i.e., the zero class; x = 0), the average lesion per DNA strand was determined as: $\lambda = -\ln A_D/A_0$.

Cardiac aconitase activity and protein analysis

Frozen left ventricular tissues (stored at -80^{o}C) were homogenized in ice-cold 10 mM Tris-KCl buffer, pH 7.4, using a polytron homogenizer. Homogenates were

centrifuged at 12,000 x g for 20 min at 4^{o}C. The supernatants were quick frozen, stored at –80^{o}C and later analyzed for aconitase activity. Aconitase activity was measured based on the formation of NADPH from $NADP^{+}$ using the Bioxytech Aconitase-340 Assay (Oxis International Inc., Foster City, CA). Total protein content was analyzed using Coomassie Plus Protein Assay Kit with bovine serum albumin as a standard (Pierce, Rockford, IL).

Statistical analysis

Statistical analysis of BALF cells and aconitase data was done by a two-way analysis of variance (ANOVA) with treatment as one factor and time as the other using SigmaStat software, version 3.5 (SPSS Inc., Chicago, IL) whereas one-way analysis of variance was performed for cardiac mitochondrial DNA damage data. One control animal at 16 week was excluded from the study as it demonstrated pulmonary complications immediately following the first intratracheal instillation of saline as determined by measurement of breathing parameters. Pair wise comparisons between groups were made using Fisher's least significant difference (LSD) test. The accepted level of significance was $p < 0.05$.

Analysis of microarray gene-chip data

Affymetrix CEL Data files were imported into R, an open source statistical scripting language (http://www.R-project.org; Ihaka and Gentleman 1996) that was used in conjunction with the Bioconductor project (http://www.bioconductor.org; Gentleman et al. 2004). Normalized values with RMA background correction, quantile normalization and median polish were calculated with the R/bioconductor package AffylmGUI (Wettenhall et al. 2006). AffylmGUI allows a graphical user interphase for the analysis

of Affymetrix microarray GeneChips using the limma package (linear modes for microarray data) in R (Smyth 2005). A linear model was fit to the data and used to average data between replicate arrays and to identify variability between them. Contrasts between groups were used to generate P-values, moderated t statistics, Empirical Bayes statistics, and M values [$\log_2$ (ratio)]. The following contrasts were made for this study; MSH/saline, PM-DH/saline, Zn/saline, Zn/MSH. Probe sets with a P-value ($p< 0.05$) were judged by the limma package to be differentially expressed within group contrasts. This list was further filtered by fold change ($1.25< X <0.75$).

The microarray data discussed in this manuscript were deposited in the Gene Expression Omnibus website (GEO; http://www.ncbi.nlm.nih.gov/geo/; Edgar et al. 2002) and are accessible through GEO series under the accession number GSE6541.

The heat map for the differentially expressed gene list was generated with the use of The Institute for Genomic Research MultiExperiment viewer (TIGR MeV, version 3.0) (Saeed et al. 2003). Differentially expressed genes for MSH, PM-HD, and zinc sulfate relative to saline and zinc relative to MSH were identified, and grouped manually into functional categories. These genes are listed in the Supplemental Material, tables 1-3. The Supplemental Material Table 4 depicts fold change in zinc exposed rats normalized to MSH. The Venn diagram was derived using GeneSpring 7 software (Agilent Technologies, Palo Alto, CA) for MSH, PM-HD and zinc (normalized to saline) to determine commonalities and differences in changed genes between groups.

Results

Pulmonary injury as determined by bronchoalveolar lavage

Chronic pulmonary inflammation can influence cardiac physiology. In order to determine the extent of pulmonary inflammation in each exposed group, BALF total cells were analyzed. Weekly instillations of MSH, PM suspensions (PM-HD and PM-LD), PM-L, and zinc, all caused an increase in BALF total cells (Figure 1). The inflammation caused by PM-HD at 8 or 16 weeks was greatest. Exposure to soluble metal-free MSH also increased BALF cells significantly but to a lesser extent than PM-HD. The degree of inflammation was greater in all the 8 weeks post IT challenges compared to the 16 weeks time point. This was expected, as the 16 weeks animals received half the concentration of each PM components on a weekly basis when compared to the 8 weeks rats. Although the increases in total cells were apparent at 16 weeks in PM-LD, PM-L, and zinc exposed rats, they were statistically insignificant.

Cardiac histopathology

In the present study the lesions were characterized by foci of myocardial degeneration, inflammation, and fibrosis. These foci were randomly distributed although frequently found at subepicardial or epicardial locations (Figure 2). A careful evaluation of serial sections of myocardial tissues demonstrated no lesions in saline controls at 8 weeks, but 2 out of 7 control animals did show mild myocardial degeneration and inflammation at 16 weeks. The photomicrographs for all 16 weeks exposure groups are depicted in the Supplemental Material, Figure1. In general, the exposure of rats to MSH, PM-HD and PM-LD, PM-L, and zinc sulfate, all caused small increases in lesion severity relative to saline controls at both time points. The lesion severity was statistically significant in 8 weeks rats exposed to MSH or PM-HD suspension (Figure 3). However,

because of low incidence of lesions in control rats at 16 weeks, the differences between groups were not statistically significant. Also, because of the limited group size (n=8 for all groups), statistical significance could not be reached across the exposure regimens. Careful evaluation of the location of the lesions in each exposure group revealed no clear distributional differences between groups. Thus, based on a histopathological evaluation, it was difficult to identify the difference in lesion severity between different exposure conditions.

Cardiac mitochondrial DNA damage

The mitochondrial DNA damage in left ventricular tissues from rats exposed for 8 weeks was analyzed using Q-PCR. The rationale of the Q-PCR assay is that the damage in either mitochondrial or nuclear DNA reduces the amplification efficiency of the template leading to reduction of PCR product with the damaged template. The DNA damage is calculated as discussed in methods section. The results show that the rats exposed to PM-HD and zinc sulfate had significantly increased mitochondrial DNA damage compared to saline (Figure 4). Rats exposed to PM-L also indicated an increase in lesions compared to saline or PM without zinc (MSH), but it was not statistically significant. MSH did not cause mitochondrial DNA damage.

Cardiac aconitase activity

Two isoforms of aconitase exist in the cell. One is cytosolic and other is mitochondrial. The iron-sulfur clusters of both aconitase isoforms are prone to inactivation by oxidative stress (Cairo et al. 2002; Tong and Rouault 2007), and thus, their activity analysis have been extensively used to demonstrate oxidative stress. We determined total aconitase activity at both time points in cardiac tissue homogenates, which included cytosolic plus mitochondrial isoform. There was a small but statistically

significant inhibition of aconitase activity in rats exposed to zinc and PM-L in the 8 weeks group. Although a trend of inhibition was apparent, aconitase activity was not significant in other groups when compared to saline (Figure 5).

Cardiac gene expression

In order to understand mechanistic differences between cardiac effects of solid PM without zinc and zinc sulfate, we performed microarray analyses of cardiac tissues from saline, MSH, PM-HD and zinc sulfate exposed rats at the 8 weeks time point. Unlike cardiac gene expression changes following high dose single pulmonary exposure to zinc sulfate (Gilmour et al., 2006b), small changes in a limited number of genes were noted in the present study (Supplemental Material, Tables 1-4). Therefore, false discovery rate correction was not employed to minimize omission of the exposure-related changes. However, we did apply a fold change cutoff for listed genes ($1.25< x <0.75$). A Venn diagram indicating number of genes, commonly or distinctly affected by MSH, PM-HD and zinc sulfate is given in the Figure 6. Table 5 in the supplemental material provides the list of genes for each distinct sections of the Venn diagram.

A very limited number (~21 non-EST) of genes showed small increases or decreases with MSH when compared to saline controls (Figure 7; Supplemental Material, Table 1). These did not fall into one specific functional category. It should be noted that some of these changes might have occurred by chance alone. Genes that were commonly affected in MSH and PM-HD containing zinc (Figure 6) included cyclin-dependent kinase inhibitor 1A (decrease), protein tyrosine phosphatase receptor type M (increase), and a gene similar to hypothetical predicted protein CG003 (increase).

The genes whose expression changed in response to PM-HD exposure were very different from those affected by MSH exposure (Figure 7; Supplemental Material,

Table 2). Surprisingly, while more non-EST genes were affected (~27) by PM–HD when compared to changes seen in MSH group (~21), the number of genes showing expression changes within this group was much smaller than the those provoked by zinc exposure (Figure 7). However, some genes changed in PM-HD were same as those affected by zinc (Figure 6), suggesting that those genes are changed as a result of exposure to zinc.

Table 3 of the Supplemental Material lists genes that were affected by zinc as compared to saline controls. The number of genes affected by zinc was much greater than that changed by MSH or PM-HD suspension (~70). These genes were grouped into 4 functional categories--cell signaling, cell cycle and growth, mitochondrial fatty acid metabolism, and oxidative stress/inflammation. Although the fold changes in expression are modest, they are consistent with the known physiological role of zinc. The small magnitude of change in gene expression in this study may be due to the episodic, lower dose exposure paradigm rather than a single high concentration zinc exposure in our previous study (Gilmour et al. 2006a; 2006b). Also, the time point of 48 h after final instillation used in this study may have allowed reversal of some acute zinc effects.

Our goal was to investigate the role of PM-associated zinc; therefore, we normalized the expression values of zinc sulfate to the MSH, a PM without zinc (McGee et al. 2003). When the zinc values were normalized to MSH, a greater number of genes than those induced by zinc/saline (Supplemental Material, Table 4) were found to be differentially expressed, suggesting that the types of expression changes in the heart were very different between soluble zinc and PM or MSH. A variety of genes involved in the acute phase response and oxidative stress were affected, including heat shock 70kD protein 1A, and predicted heat shock protein 90kDa protein 1, which may explain

the noted cardiac mitochondrial DNA damage. Metallothionein gene expression was induced, which is consistent with our previous study (Gilmour et al. 2006b), although the increase was small and statistically insignificant (therefore, not included in gene list tables). Further, similar to the data tabulated in Table 3 of the Supplemental Material, a variety of genes involved in cell signaling, cell cycle and growth, ion transport, mitochondrial fatty acid metabolism, and oxidative stress were consistently affected when zinc-induced expression was normalized to MSH (grouped into 5 functional categories). Only a few genes appeared down regulated in the hearts of zinc-exposed rats, e. g., cyclin D1, fatty acid binding protein 3 etc.

Discussion

Zinc is a ubiquitous component of ambient PM, often second in abundance to iron. Based on our previous study demonstrating cardiac pathology in rats inhaling zinc-containing particles (Kodavanti et al. 2003), we postulated that soluble zinc is one of the causative components of inhaled PM responsible for cardiac effects. To address this hypothesis, we intratracheally exposed rats to PM with or without its zinc constituents or to zinc alone once per week for 8 or 16 consecutive weeks. We report here that increased BALF cell numbers and cardiac pathology were evident in all rats exposed for 8 or 16 weeks to PM, with or without soluble zinc and to soluble zinc. However, only the rats exposed to soluble zinc or zinc containing PM demonstrated mitochondrial DNA damage. Aconitase activity was inhibited slightly, but significantly in rats exposed to zinc or zinc containing PM leachate. Furthermore, gene expression profiles of the cardiac tissue demonstrated small but significant changes in expression pattern in rats exposed to zinc but not in rats exposed to MSH, a PM without zinc. These changes were reflective of altered cell signaling, cell cycle and growth, oxidative stress, inflammation, ion channels function, protease/antiprotease balance, and mitochondrial fatty acid metabolism. Thus, it appears that while insoluble PM may induce a small degree of cardiac pathology, perhaps via chronic pulmonary inflammation, soluble zinc may contribute to cardiac injury via its effects on gene expression, and mitochondrial DNA damage.

Cardiac injury from pulmonary PM exposure can occur via systemic endothelial activation and/or lung inflammation (Godleski 2006). Cardiac effects can also occur directly by PM-associated metals (Gilmour et al. 2006b). It can not be ascertained from our present study the full spectrum of direct effects related to soluble components such

as zinc, since cardiac pathology and marked pulmonary inflammation were evident in all groups including animals exposed to MSH without soluble zinc (McGee et al., 2003). However, distinct gene expression changes together with small mitochondrial aconitase inhibition and DNA damage occurred only in zinc-exposed rats. Based on this evidence, we postulate that presence of pulmonary inflammation may lead to pathology in the heart, whereas zinc may affect broader physiological processes at multiple levels without apparent lesion development. The data supports the hypothesis that zinc may be responsible, at least in part, for the PM cardiac effects.

In the previous study, we demonstrated that pulmonary exposure to a large bolus of zinc resulted in ~20% increase in circulating zinc at 1 hour following exposure, whereas cardiac effects were manifested at 4 and 24 h time points, when circulating zinc had returned to control levels (Gilmour et al. 2006b). No net increase in cardiac zinc was noted in that study. Also, the resulting increase in plasma zinc was transient, ultimately with the accumulation of zinc in the liver. Interestingly, at 24 h, copper and selenium in the liver also increased suggesting that pulmonary zinc exposure can result in systemic imbalance of other essential metals (Gilmour et al. 2006b). Zinc has been shown to interact with other essential metals in the body and in the heart (Powell et al. 1999). Thus, the cardiac effects might be due to acute transient elevations in circulating zinc leading to disturbances in essential metal balance immediately following each instillation. Since zinc is best known to bind to metallothionein protein, and a massive induction of this gene has been noted in the lung immediately following instillation of soluble zinc (Giilmour et al. 2006b), it would appear that pulmonary zinc might be carried to circulation in its protein bound form (Agte and Nagmote 2004); however, not stored in the heart.

At high levels, zinc is known to inhibit mitochondrial respiration in rat liver but not cardiac tissues (Ye et al. 2001). An increase in circulating zinc, however, has been shown to cause a number of cardiac mitochondrial effects (Gilmour et al. 2006b). It is not known if acute pulmonary zinc exposure inhibits cardiac aconitase activity, however, zinc transfer from metallothionein to cardiac mitochondrial aconitase has been noted in mouse heart extracts (Feng et al. 2005). Further, Inhibition of cardiac aconitase activity in the present study may involve inactivation of cytosolic and/or mitochondrial isoforms secondary to zinc-induced oxidant production. Aconitase is highly susceptible to inhibition by oxidative stress and has been extensively used as a marker to demonstrate production of free radicals in tissues (Tong and Rouault 2007). The probable involvement of increased oxidant production is also reflected in the increase in mitochondrial DNA damage reported here. The mechanism is not clear because zinc is present in the regulatory domain of numerous proteins within cytosol and mitochondria (Maret 2001, 2004; Sharpley and Hirst 2006). Thus, perturbation of more than one metal-protein coordinations is likely to be involved. Evidence of zinc-induced oxidative stress is seen in the neuronal release of zinc and associated degenerative changes in the brain (Dineley et al. 2003).

While modest, changes in the gene expression provided an important mechanistic insight into potential long-term effects of zinc on the myocardium. Only a few changes occurred in the pattern of cardiac gene expression in MSH-exposed rats, whereas relatively large changes were noted in rats exposed to zinc sulfate. This suggests that a small increases in cardiac lesions may be independent of zinc-specific effect on cardiac gene expression. It is likely that effects on gene expression caused by zinc may not result in chronic cardiac pathology in the present study. It is not clear why

exposure of rats to whole PM suspension (PM-HD) containing soluble zinc did not alter as many genes as those altered by zinc. However, it is noteworthy that some of the genes induced by the PM-HD were also induced by zinc suggesting a zinc-specific effect. It is also possible that the presence of small amounts of nickel (Kodavanti et al. 2002) and also solid PM may interfere with PM-zinc absorption, and metal-metal interactions may limit zinc bioavailability. We have previously noted that the toxicity of one individual metal may be reduced in the presence of other metals (Kodavanti et al. 1997) and interactions of zinc and copper are well documented (Klevay 2000; Powell et al. 1999). The association between PM-induced long-term pulmonary inflammation and increases in the background cardiac lesions need to be further examined.

The changes in the cardiac gene expression pattern from pulmonary exposures to soluble zinc seen in the present study were expected based on the known biological role of zinc in cell growth, metabolism, cell signaling and mitochondrial respiration (Maret, 2006; Samet et al. 2003; Ye et al. 2001). Although the direct effect of zinc on each of the genes whose expression was altered has not been reported in the literature, the functional grouping of these genes suggests multiple sites of action. For example, numerous genes for proteins, that regulate cell signaling, such as kinases and phosphatases were stimulated, while the epidermal growth factor receptor gene was down regulated. In isolated cells, zinc in protein-free medium is known to modulate phosphorylation of these proteins (Samet et al. 2003). Unlike down regulation of mRNA expression of phosphatases following one bolus and acute exposure (Gilmour et al. 2006b), in the present study episodic long-term exposure to zinc led to upregulation of these genes which may reflect the chronic outcome of multiple episodic exposures.

Excess zinc in isolated cells has been shown to regulate calcium, sodium and potassium channels (Aedo et al. 2007; Graff et al. 2004; Maret 2001). Rats exposed to zinc but not MSH showed modulation of genes that either forms these ion channels or their regulatory proteins (apparent when zinc data were normalized to MSH). Interestingly, induction as opposed to the previously noted bi-directional effect was noted following acute zinc exposure (Gilmour et al. 2006b). This suggests compensatory regulation of cellular homeostasis by activation of ion transport mechanisms. Chronic effects of excess zinc on calcium, potassium, and sodium channels have not been well investigated and may affect cardiac conductance properties. In our subsequent study, heart rates were analyzed in rats exposed to MSH, PM-HD and zinc (Rowan et al., 2005). Zinc and PM-HD but not MSH caused a small but acute drop in heart rate which was reversible within one day. Excess zinc suppresses rat myocyte beat frequency in vitro (Graff et al. 2004) and also heart rate in isolated guinea pigs hearts (Evangelou and Kalfakakou 1993). Thus, it is likely that zinc has acute cardiac physiological effects; however, the long-term effects remain unclear.

Excess zinc modulates mitochondrial respiration in rat liver likely via its effect on electron transport (Kuznetsova et al. 2005). In the present study, zinc exposure did not cause gene expression changes in components of cardiac electron transport proteins, however, it did increase the expression of genes involved in mitochondrial fatty acid metabolism. It is possible, therefore, that an increase in fatty acid metabolism could cause an increase in free radical production within mitochondria and lead to mitochondrial DNA damage and inhibition in aconitase activity following multiple episodic exposures.

The suppression of gene expression regulating heat shock proteins, stimulation of matrix metalloproteinase inhibitors, changes in hypoxia-regulated genes, and upregulation of oxidative stress sensitive genes observed in our study may imply increased free radical production. The excess zinc has been shown to affect these processes in a variety of cell types (Chun et al. 2001; Larbi et al. 2006; Puerta et al. 2006). This zinc-induced oxidative stress response is contrary to its commonly reported cardioprotection, and its antioxidant function via metallothionein (Kang 1999; Maret 2006; Satoh et al. 2000).

In summary, we report here that protracted episodic intratracheal exposures to PM with or without soluble zinc resulted in chronic lung inflammation. The inflammatory response in the lung was associated with modest increases in lesion severity in the heart. However, although small, cardiac mitochondrial DNA damage, inhibition of aconitase activity, and changes in cardiac gene expression patterns were observed only in rats exposed to zinc, or (to some extent) zinc containing PM. These findings suggest that long-term inhalation of urban PM containing high levels of zinc may be linked to increased risk of cardiac morbidity.

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Table 1. Experimental design depicting group designation and weekly exposure concentrations of insoluble (solid) PM mass and soluble zinc sulfate.

Animal Group #	Test Material	Group designation	8 Weeks: PM-solid instillation, mg/kg/wk	8 Weeks: Soluble zinc instillation, μg/kg/wk	16 Weeks: PM-solid instillation, mg/kg/wk	16 Weeks: Soluble zinc instillation, μg/kg/wk
1	Saline	Saline	0.00	0.00	0.00	0.00
2	Mount. St Helen's Ash	MSH	4.60	0.00	2.30	0.00
3	Whole suspension of oil combustion PM at high concentration	PM-HD	4.60	66.8	2.30	33.4
4	Whole suspension of oil combustion PM at low concentration	PM-LD	2.30	33.4	1.15	16.7
5	Saline-leachable fraction of PM high concentration suspension	PM-L	0.00	66.8	0.00	33.4
6	Zinc sulfate, $7H_2O$	Zn	0.00	66.8	0.00	33.4

Figure Legends

Figure 1. Pulmonary toxicity of soluble and solid PM components as determined by recovery of cells in bronchoalveolar lavage fluid (BALF). Group designations are as follows: saline, control; MSH, Mount St. Helens Ash; PM-HD, whole particle suspension instilled at high concentration; PM-LD, whole particle suspension instilled at low concentration; PM-L, saline-leachable fraction of PM; and Zn, zinc sulfate. Note that 8 weeks rats received double the dose of each PM components given to 16 weeks. Values represent mean ± standard error (n=7-8 rats per group). Asterisks (*) indicate $P \leq 0.05$ when compared to saline control. Within group comparison indicated significant differences ($P \leq 0.05$) at 8 wks: PM-HD vs. PM-LD, PM-HD vs. PM-L; PM-HD vs. MSH; PM-HD vs. Zn; Zn vs. PM-LD; MSH vs. PM-LD; MSH vs. PM-L and PM-L vs. PM-LD; and at 16 wks: PM-HD vs. PM-LD, PM-HD vs. PM-L; PM-HD vs. Zn; PM-HD vs. MSH; MSH vs. PM-L; MSH vs. Zn.

Figure 2. Cardiac histopathological lesions in rats intratracheally exposed to saline or various fractions of PM for 8 consecutive weeks. The lesion distribution was focal and not widespread (Severity grades 1-2). No specific region appeared more affected over other by PM with or without zinc. Lesions were noted in all exposure groups except for saline controls. Arrows indicate areas of myocardial degeneration and chronic inflammation. In most cases lesions were apparent in subepicardial region; however, no exposure group-related pattern for lesion location was evident. Saline=control; MSH=Mount St. Helen's ash; PM-HD and PM-LD=high and low concentration particle exposure groups, respectively; PM-L=PM leachate; Zn=Zinc sulfate.

Figure 3. Semiquantitative grading of the extent of lesions within myocardium of rats exposed to different PM fractions. Group designations are as follows: saline, control; MSH, Mount St. Helens Ash; PM-HD and PMLD, whole particle suspension instilled at high and low concentrations, respectively; PM-L, saline-leachable fraction of PM; and Zn, zinc sulfate. Pathology severity score of 0=none, 1=minimal, 2=moderate, 3=marked, and 4=severe lesions were employed. Mean severity of lesions was calculated by adding the severity score for all animals within the group and then dividing by total number of animals. Values represent mean ± standard error (n=7-8 rats per group). Asterisks (*) indicate P≤0.05 when compared to saline control.

Figure 4. Cardiac mitochondrial DNA damage following eight weekly exposures to solid PM or soluble components in rats. Group designations are as follows: saline, control; MSH, Mount St. Helens Ash; PM-HD, whole particle suspension instilled at high concentration; PM-LD, whole particle suspension instilled at low concentration; PM-L, saline-leachable fraction of PM; and Zn, zinc sulfate. Note that because of sample-to-sample variation, the only group reached statistical significance was zinc sulfate, although the trend was consistent in other groups exposed to PM containing water-soluble zinc. Values represent mean ± standard error (n=8 rats per group). Note that control values are normalized to zero. Asterisks (*) indicate P≤0.05 when compared to saline control.

Figure 5. Cardiac tissue total aconitase activity in rats exposed to soluble or solid PM components for 8 or 16 weeks. Group designations are as follows: saline, control; MSH, Mount St. Helens Ash; PM-HD, whole particle suspension instilled at high concentration;

PM-LD, whole particle suspension instilled at low concentration; PL-L, saline-leachable fraction of PM devoid of solid insoluble components; and Zn, zinc sulfate. Zinc concentration in PM-HD, and PM-L and Zn groups is same. Note that rats received same dose of PM or other components for 8 and 16 weeks. Values represent mean ± standard error (n=8 rats per group). Asterisks (*) indicate P≤0.05 when compared to saline control.

Figure 6. A Venn diagram depicting differences and commonalities in number of genes expressed by given exposure condition. Differentially expressed genes for MSH, PM-HD (whole particle suspension), and zinc sulfate relative to saline were used in developing a Venn diagram. A list of genes for each distinct sections of the Venn diagram is provided in the supplemental material Table 5.

Figure 7. Heatmap showing differential gene expression as fold change intensities from group contrasts with saline control. Red and green color intensities indicate fold change increases and decreases, respectively, in gene expression (expressed as $\log_2$). Differentially expressed genes for MSH, PM-HD (whole particle suspension), and zinc sulfate relative to saline were grouped manually into functional categories.

Figure 1

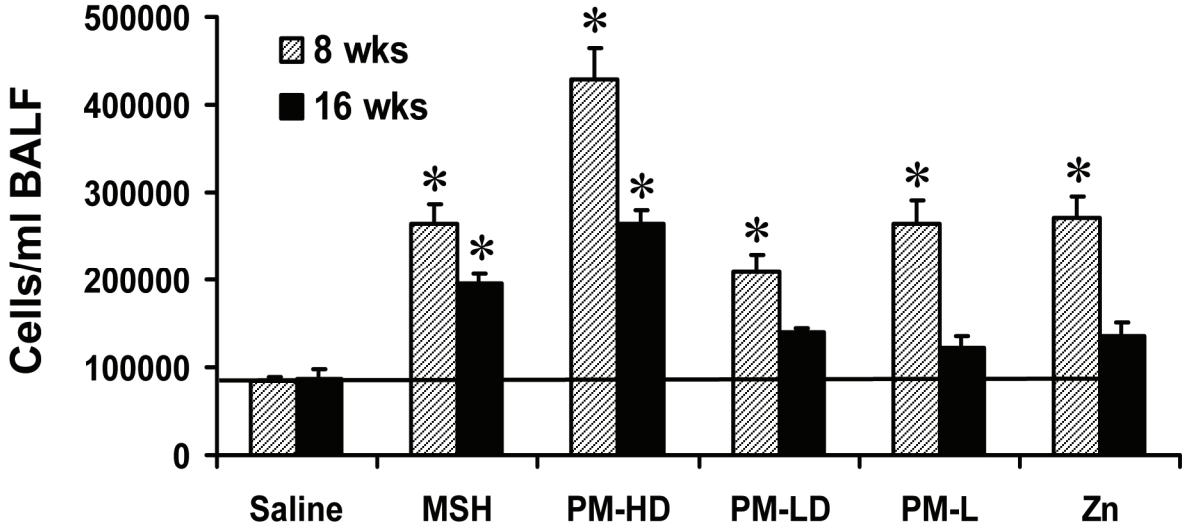

Cells/ml BALF
500000
400000
300000
200000
100000
0
8 wks
16 wks
*
Saline
MSH
PM-HD
PM-LD
PM-L
Zn

Figure 2

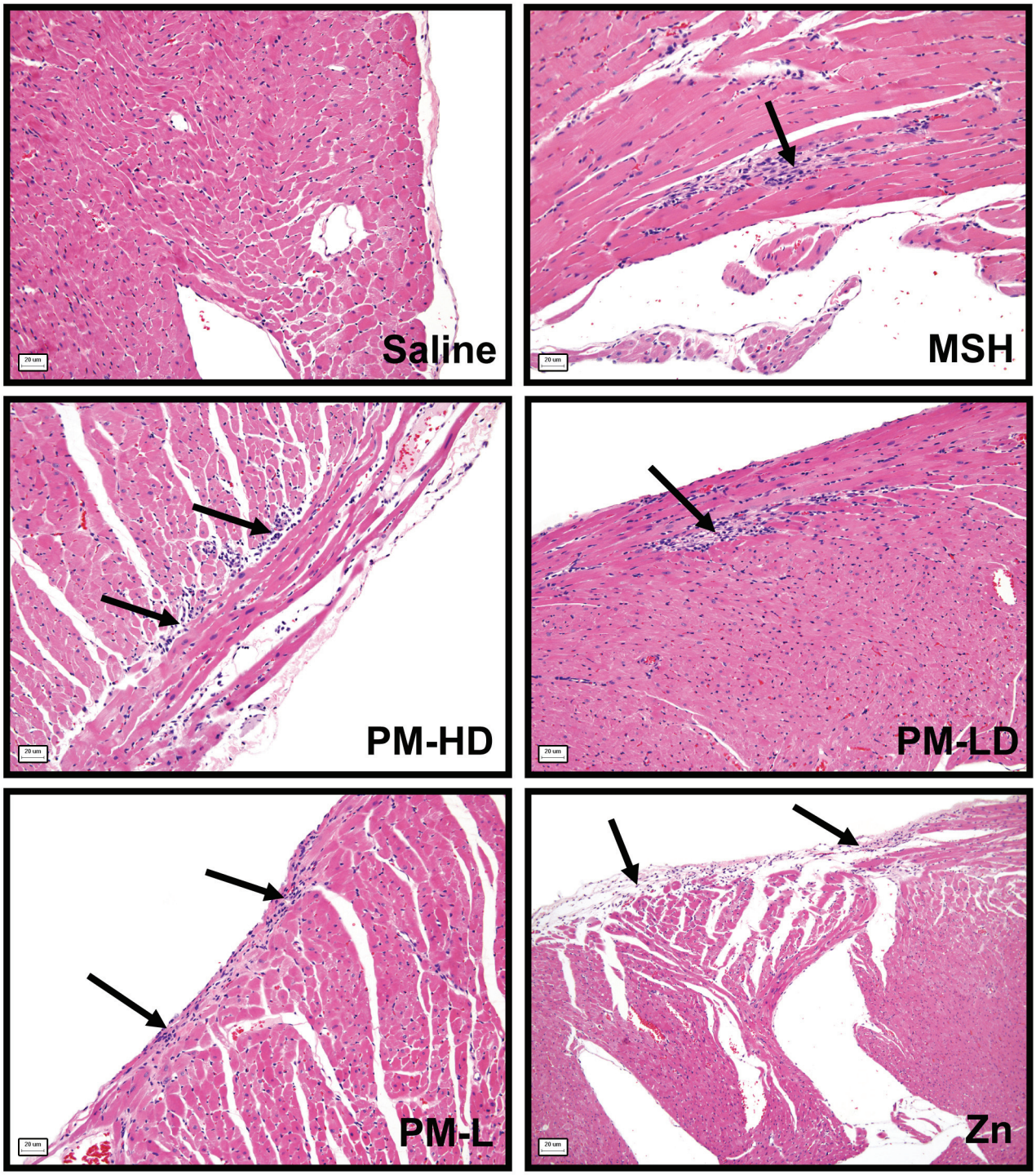


Figure 3

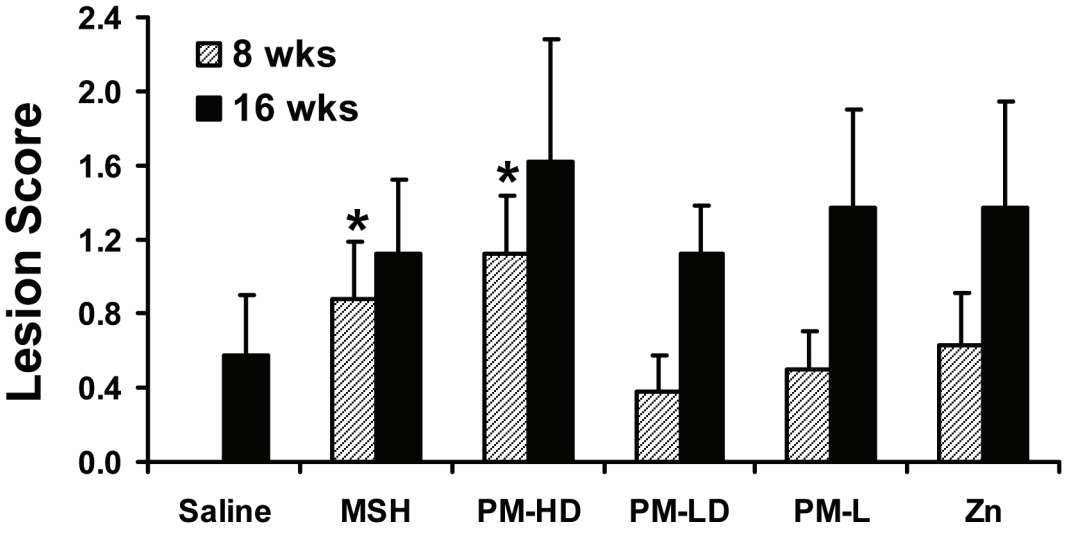

Lesion Score
2.4
2.0
1.6
1.2
0.8
0.4
0.0
8 wks
16 wks
*
*
Saline
MSH
PM-HD
PM-LD
PM-L
Zn

Figure 4

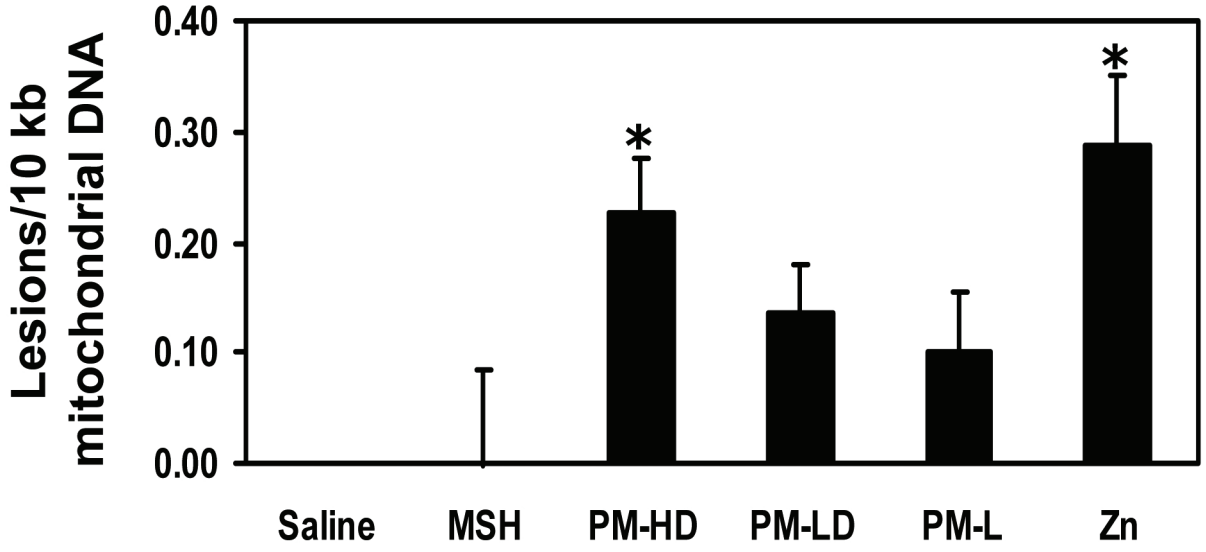

Lesions/10 kb mitochondrial DNA
0.40
0.30
0.20
0.10
0.00
*
*
Saline
MSH
PM-HD
PM-LD
PM-L
Zn

Figure 5

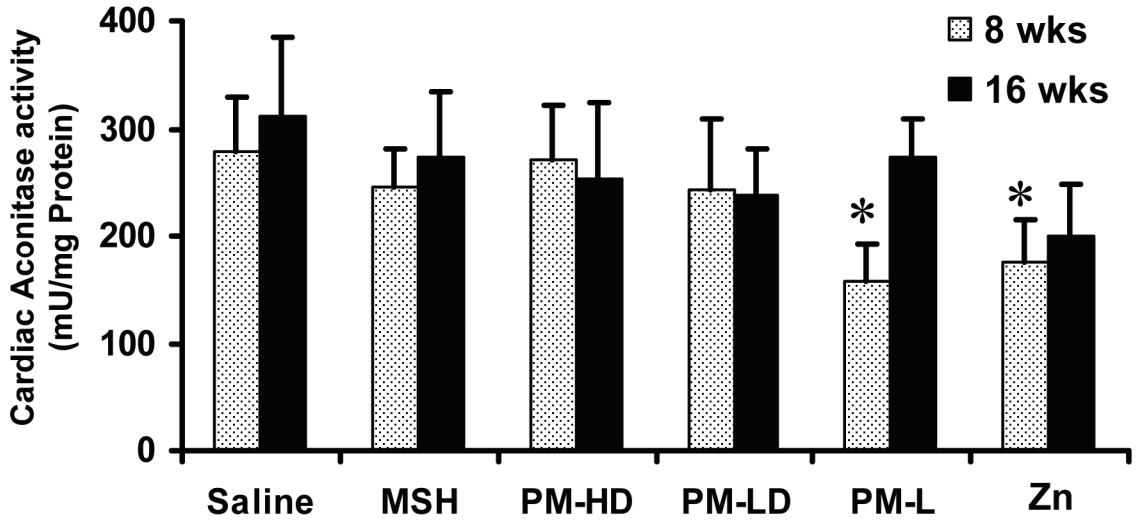

Cardiac Aconitase activity
(mU/mg Protein)
400
300
200
100
0
8 wks
16 wks
*
*
Saline
MSH
PM-HD
PM-LD
PM-L
Zn

Figure 6

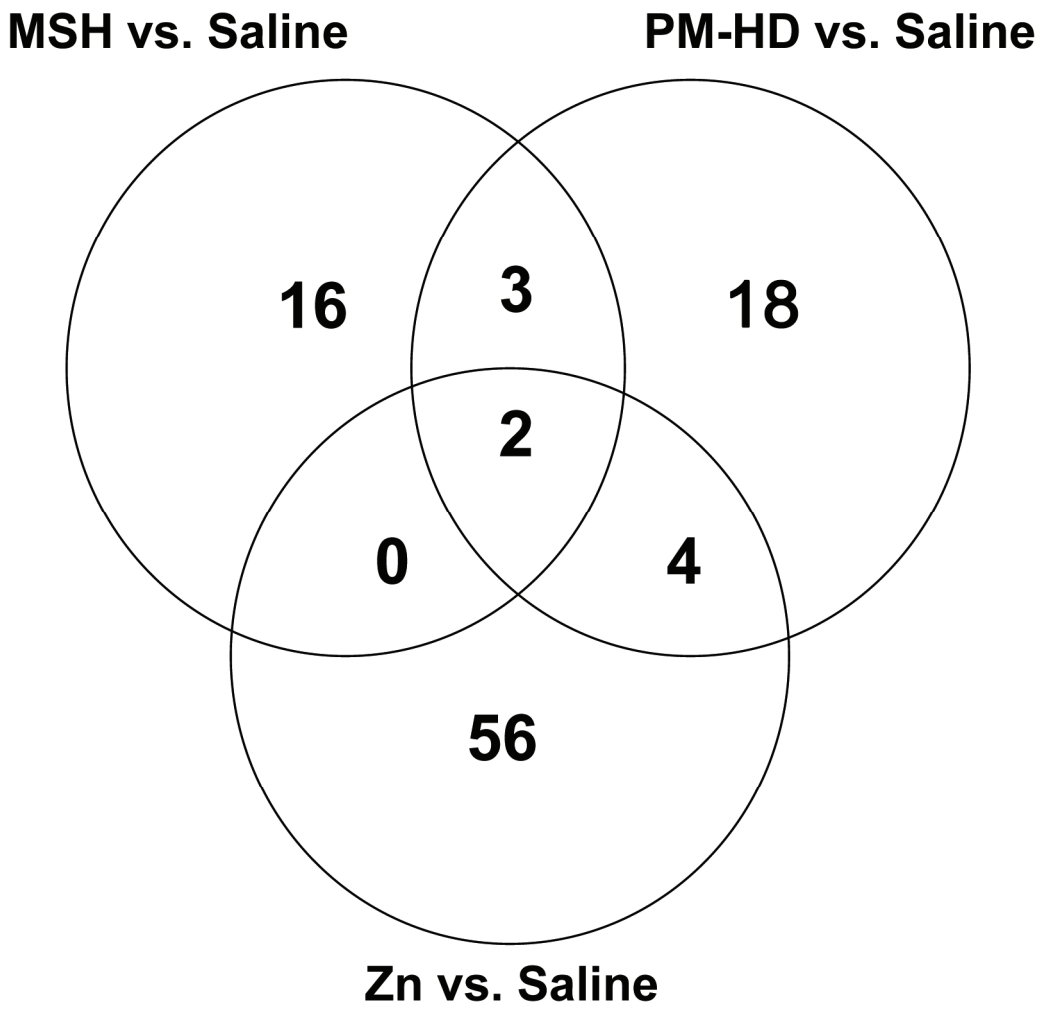
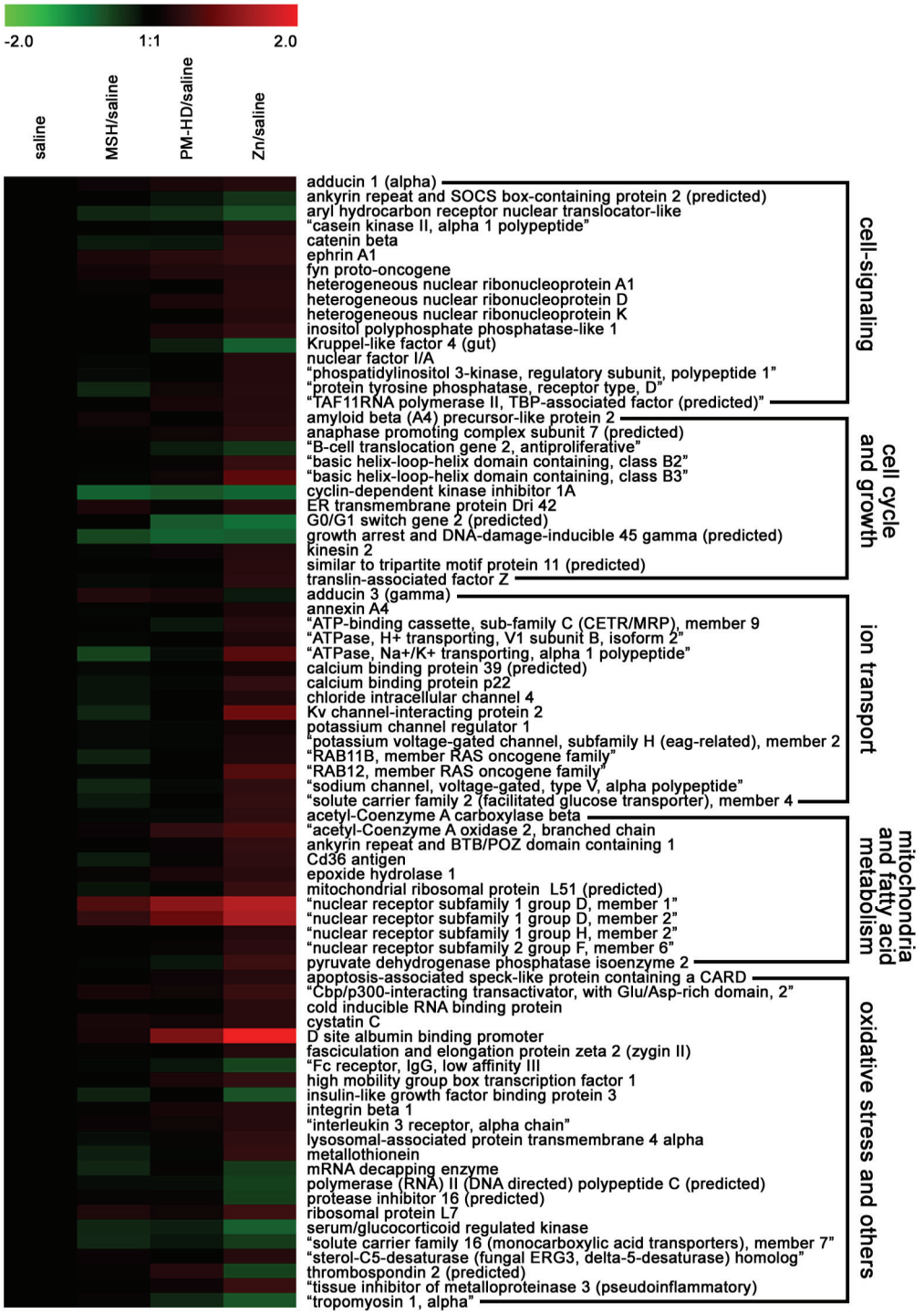


Figure 7


-2.0
1:1
2.0
saline
MSH/saline
PM-HD/saline
Zn/saline
adducin 1 (alpha)
ankyrin repeat and SOCS box-containing protein 2 (predicted)
aryl hydrocarbon receptor nuclear translocator-like
"casein kinase II, alpha 1 polypeptide"
catenin beta
ephrin A1
fyn proto-oncogene
heterogeneous nuclear ribonucleoprotein A1
heterogeneous nuclear ribonucleoprotein D
heterogeneous nuclear ribonucleoprotein K
inositol polyphosphate phosphatase-like 1
Kruppel-like factor 4 (gut)
nuclear factor I/A
"phospatidylinositol 3-kinase, regulatory subunit, polypeptide 1"
"protein tyrosine phosphatase, receptor type, D"
"TAF11RNA polymerase II, TBP-associated factor (predicted)"
amyloid beta (A4) precursor-like protein 2
anaphase promoting complex subunit 7 (predicted)
"B-cell translocation gene 2, antiproliferative"
"basic helix-loop-helix domain containing, class B2"
"basic helix-loop-helix domain containing, class B3"
cyclin-dependent kinase inhibitor 1A
ER transmembrane protein Dri 42
G0/G1 switch gene 2 (predicted)
growth arrest and DNA-damage-inducible 45 gamma (predicted)
kinesin 2
similar to tripartite motif protein 11 (predicted)
translin-associated factor Z
adducin 3 (gamma)
annexin A4
"ATP-binding cassette, sub-family C (CETR/MRP), member 9
"ATPase, H+ transporting, V1 subunit B, isoform 2"
"ATPase, Na+/K+ transporting, alpha 1 polypeptide"
calcium binding protein 39 (predicted)
calcium binding protein p22
chloride intracellular channel 4
Kv channel-interacting protein 2
potassium channel regulator 1
"potassium voltage-gated channel, subfamily H (eag-related), member 2
"RAB11B, member RAS oncogene family"
"RAB12, member RAS oncogene family"
"sodium channel, voltage-gated, type V, alpha polypeptide"
"solute carrier family 2 (facilitated glucose transporter), member 4
acetyl-Coenzyme A carboxylase beta
"acetyl-Coenzyme A oxidase 2, branched chain
ankyrin repeat and BTB/POZ domain containing 1
Cd36 antigen
epoxide hydrolase 1
mitochondrial ribosomal protein L51 (predicted)
"nuclear receptor subfamily 1 group D, member 1"
"nuclear receptor subfamily 1 group D, member 2"
"nuclear receptor subfamily 1 group H, member 2"
"nuclear receptor subfamily 2 group F, member 6"
pyruvate dehydrogenase phosphatase isoenzyme 2
apoptosis-associated speck-like protein containing a CARD
"Cbp/p300-interacting transactivator, with Glu/Asp-rich domain, 2"
cold inducible RNA binding protein
cystatin C
D site albumin binding promoter
fasciculation and elongation protein zeta 2 (zygin II)
"Fc receptor, IgG, low affinity III
high mobility group box transcription factor 1
insulin-like growth factor binding protein 3
integrin beta 1
"interleukin 3 receptor, alpha chain"
lysosomal-associated protein transmembrane 4 alpha
metallothionein
mRNA decapping enzyme
polymerase (RNA) II (DNA directed) polypeptide C (predicted)
protease inhibitor 16 (predicted)
ribosomal protein L7
serum/glucocorticoid regulated kinase
"solute carrier family 16 (monocarboxylic acid transporters), member 7"
"sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog"
thrombospondin 2 (predicted)
"tissue inhibitor of metalloproteinase 3 (pseudoinflammatory)
"tropomyosin 1, alpha"
cell-signaling
cell cycle and growth
ion transport
mitochondria and fatty acid metabolism
oxidative stress and others